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(54) Title: MICROCRYSTALLINE CELLULOSE AS AN IMMUNE ADJUVANT

(57) Abstract

The present invention relates to compositions that comprise microcrystalline cellulose as an immune adjuvant, and to methods of inducing immunity to pathogens that comprise the administration of such compositions. It is based, at least in part, on the discovery that microcrystalline cellulose exhibits immune adjuvant properties superior to those of conventional adjuvants.

MICROCRYSTALLINE CELLULOSE AS AN IMMUNE ADJUVANT

The present application is a continuation-in-part of U.S. Application No. 07/971,161 filed November 3, 1992 the complete disclosure of which is incorporated by reference herein.

1. INTRODUCTION

The present invention relates to compositions that comprise microcrystalline cellulose as an immune adjuvant, and to methods of inducing immunity to pathogens that comprise the administration of such compositions. It is based, at least in part, on the discovery that microcrystalline cellulose exhibits immune adjuvant properties superior to those of conventional adjuvants.

2. BACKGROUND OF THE INVENTION

2.1. IMMUNE ADJUVANTS

An immune adjuvant is a substance which, when administered in conjunction with a particular immunogenic substance (the "immunogen"), enhances the response of the immune system toward the immunogen (Benjamini and Leskowitz, 1988, in "Immunology: A Short Course", Alan R. Liss, Inc., New York, p. 39). Widely used adjuvants include Freund's complete adjuvant, a water-in-oil emulsion containing killed *Mycobacteria*; Freund's incomplete adjuvant, which differs from Freund's complete adjuvant by the absence of *Mycobacteria*; bacillus Calmette-Guerin ("BCG"), an attenuated *Mycobacterium*; *Corynebacterium parvum*; *Bordetella pertussis*; lipopolysaccharide; muramyldipeptide; and alum (*Id*.).

Many of these adjuvants exhibit disadvantages with regard to safety or efficacy. For example,

Freund's complete adjuvant is highly effective in enhancing the immune response but is not acceptable for use in humans or domestic animals due, in part, to the presence of non-degradable mineral oil and the necrotic side-effects of the Mycobacteria. Incomplete Freund's adjuvant is safer, but less effective. Alum, the only adjuvant currently approved for human use, has been incorporated into influenza, diphtheria, and tetanus vaccines, but has failed to augment immunity in several cases, including whooping cough and typhoid fever vaccine (Butler et al., 1962, Lancet 2:114-115, Cvgetanovic and Vemra, 1965, Bull. W.H.O. 32:29-36).

2.2. MICROCRYSTALLINE CELLULOSE

Cellulose is one of the most widely used materials in the textile, paper, food and pharmaceutical industries. Various forms of cellulose are used routinely as pharmaceutical excipients. These include: (a) powdered cellulose, used as a capsule and tablet diluent; (b) microcrystalline cellulose, also used as a capsule and tablet diluent, a disintegrant, and a suspension agent or viscosity increasing agent; (c) cellulose acetate, used for the same purposes as microcrystalline cellulose; (d) cellulose acetate phthalate and hydroxypropyl methycellulose phthalate, used as enteric coating films; (e) hydroxypropyl methycellulose and methyl cellulose, used as viscosity increasing agents, tablet binders and coating agents; and (f) hydroxy ethyl cellulose, used as a viscosity increasing and coating agent.

Cellulose is a polymer composed of glucose residues in β (1→4) linkage. The empirical formula is $(C_6H_{10}O_5)_n$, where n is 1,500 for powdered cellulose (MW = approx. 243,000), and 220 for microcrystalline cellulose (MW = approx. 36,000). Microcrystalline

cellulose is a white, odorless, tasteless, crystalline powder composed of porous particles. It is insoluble in water and dilute acids. The Ph of a 12.5% suspension in water ranges from Ph 5.0 to Ph 7.0. It is available commercially as *Avicel* (FMC Corporation, Philadelphia, PA) in different average particle size grades and properties, i.e., PH-101 (50 μm), PH-102 (100 μm), PH-103 (50 μm) and PH-105 (20 μm). A number of microcrystalline cellulose derivatives, including methyl cellulose and carboxymethylcellulose, are water soluble, and two (cellulose acetate phthalate and hydroxypropyl methycellulose phthalate) are soluble at neutral and basic pH.

2.3. CELLULOSE AND THE IMMUNE SYSTEM

A number of reports have included, within their scope, both cellulose (or its derivatives) and the immune system. For example, the subcutaneous implantation of pellets of cellulose sponge cloth has resulted in local granuloma formation (Cashin et al., 1977, J. Pharm. Pharmal. 29:330-336). Cellulose sulfate, and other sulfated homopolysaccharides, have been reported to be lymphocyte mitogens (Mizumoto et al., 1988, Japan J. Exp. Med. 58:145-151). Immunogen-cellulose complexes, obtained by the covalent coupling of immunogen to suspended cellulose particles, were found to be highly effective in enhancing the antibody response toward immunogen; however, this enhancement was only achieved if immunogen was covalently coupled to the cellulose -- a noncovalently linked mixture of immunogen and cellulose was no more effective at inducing antibody formation than immunogen alone (Gurich and Korukova, 1986, J. Immunol. Meth. 87:161-167).

Immunogen immobilized on nitrocellulose paper has been found to be effective at inducing immunity toward the immunogen (Van Hamont et al., 1986, Abstracts of the Annual Meeting of the American Society of Microbiology, p. 109, Abstract E-76; Diano et al., 1987, Analyt. Biochem. 166:224-229; Nilsson et al., 1987, J. Immunol. Meth. 99:67-75; Larsson and Nilsson, 1988, Scand. J. Immunol. 27:305-309; Healy et al., 1989, Lab. Invest. 60:462-470; Coghlan and Hanausek, 1990, J. Immunol. Meth. 129:135-138). According to some of these reports, immunogen was separated from contaminating compounds by electrophoresis and blotted onto nitrocellulose paper, which was then introduced into an animal host in the form of paper strips (Nilsson et al., 1987, J. Immunol. Meth. 99:67-75; Larrson and Nilsson, 1988, Scand. J. Immunol. 27:305-309; Healy et al., 1989, Lab. Invest. 60:462-470; Coghlan and Hanausek, 1990, J. Immunol. Meth. 129:135-138). Other groups, after binding immunogen to nitrocellulose paper, sonicated the paper to reduce it to a particulate composition for administration (Van Hamont et al., 1986, Abstracts of the Annual Meeting of the American Society of Microbiology, p. 109, Abstract E-76; Diano et al., 1987, Analyt, Biochem. 166:224-229). Antibody responses toward nitrocellulose-associated immunogen were greater than antibody responses toward immunogen administered alone (Larrson and Nilsson, 1988 Scand. J. Immunol. 27:305-309).

In contrast, polylysine/carboxy-methylcellulose was found not to exhibit adjuvant activity by Levy et al. (1980, Annals New York Acad. Sci. 350:33-41) and Harrington et al. (1979, Infection and Immunity 24:160-166). Both of these reports relate to polyriboinosinic/polyribocytidylic acid (poly (I)-

poly(C)) stabilized with poly-l-lysine and carboxy-methyl-cellulose (to form poly (ICLC)). Whereas poly (ICLC) was found to enhance immune reactivity to influenza virus vaccine (Levy et al., supra) or Venezuelan equine encephalomyelitis virus vaccine (Harrington et al., supra), presumably as a result of interferon induction, polylysine/carboxymethyl-cellulose alone was found to have no immune adjuvant action (Levy et al., supra, p. 34; Harrington et al., supra, p. 162).

3. SUMMARY OF THE INVENTION

The present invention relates to compositions that comprise microcrystalline cellulose as an immune adjuvant and to methods of inducing immunity to pathogens that comprise the administration of such compositions. It is based, at least in part, on the discovery that formulations of microcrystalline cellulose-based adjuvant appear to be superior to previously known adjuvants at enhancing the antibody response toward an immunogen. The present invention also provides for non-covalently linked mixtures of microcrystalline cellulose and immunogen and for a supernatant of vacuum-dried cellulose that has adjuvant activity.

In various embodiments, the microcrystalline cellulose may be comprised in a composition which further contains other forms of cellulose and/or various diluents, binders, etc., including, but not limited to, cellulose acetate, sucrose, starch, or gelatin. The microcrystalline cellulose-based adjuvant of the invention may be administered either orally, intraperitoneally, intranasally, intravaginally, intravenously, intrathecally, by

inhalation, or intrarectally or, preferably, intramuscularly or subcutaneously.

4. DETAILED DESCRIPTION OF THE INVENTION

For purposes of clarity of description, and not by way of limitation, the detailed description of the invention is divided into the following subsections:

(i) vaccine formulations; and

(ii) methods of vaccine administration.

4.1. VACCINE FORMULATIONS

The present invention provides for compositions having immune adjuvant activity that comprise microcrystalline cellulose. The term microcrystalline cellulose, as used herein, refers to cellulose having a molecular weight of between about 30,000 and 700,000 daltons, and having a particle size less than about 250 microns. In certain embodiments, the particle size may be less than 10 microns and may be preferably between .1 and 5 microns. The term microcrystalline cellulose also refers to cellulose derivatives having a molecular weight of between about 30,000 and 700,000 daltons and having a particle size less than about 250 microns, including, but not limited to, cellulose acetate, carboxymethyl cellulose, powdered cellulose acetate phthalate, methylcellulose, ethyl cellulose and hydroxypropyl-cellulose.

In specific, non-limiting embodiments of the invention, the compositions comprise at least 2 percent and preferably at least ten percent, microcrystalline cellulose.

The compositions of the invention may further comprise non-microcrystalline forms of cellulose, such as powdered cellulose.

In addition, the compositions of the invention may comprise various substances that are commonly used in pharmaceutical compositions, including, but not limited to, sucrose, starch, gelatin, wax, flavoring agent, solvent, coloring agent, lactose, mannitol, sorbitol, acdisol, natural gums (e.g., acacia, pectin), alginate, polyvinyl pyrrolidone, polyethylene glycols, Di-Pac, EmDex, NU-TAB, oils, talc, silicas, ion exchange resins, corn syrup, and magnesium stearate. The nature of the compositions may, in part, depend on the route of administration (see infra).

In particular embodiments of the invention, microcrystalline cellulose may be obtained from, for example, FMC Corporation, Philadelphia, PA under the trade name "Avicel."

The adjuvant compositions of the invention may be used in conjunction with a wide number of immunogens including allergens, tumor antigens, immunogenic components of viruses, such as influenza virus, respiratory syncytial virus, hepatitis A, B, or C virus, HIV-1, HIV-2, herpes simplex virus, as well as immunogenic components of bacteria (e.g. tetanus toxoid or pertussis components), parasites (e.g. malaria) or cancer cells.

In specific, nonlimiting embodiments of the invention, immunogen may be combined with microcrystalline cellulose-based adjuvant to form a mixture prior to administration. For example, immunogen and adjuvant may be mixed in aqueous solution, dried under vacuum, then pulse blended. The amount of immunogen in the mixture may vary depending upon its intrinsic immunogenicity, but may preferably be between about one and ten milligrams, and more preferably be about four or five milligrams, per gram

of adjuvant composition. Alternatively, immunogen may be administered separately from adjuvant.

In one preferred, specific, nonlimiting embodiment of the invention, the composition may consist essentially of cellulose acetate, microcrystalline cellulose, sucrose, starch, and gelatin in a ratio, by weight, of 20:10:30:30:10, and may be pulse-blended as dry ingredients. In a related specific embodiment, immunogen may be added to the foregoing composition to form an immunogenic composition; for example, and not by way of limitation, formalin-inactivated influenza virus may be added to the adjuvant composition, e.g. at a concentration of about 0.4 percent by weight.

In another preferred, specific, nonlimiting embodiment of the invention, the composition may consist of microcrystalline cellulose, sucrose, starch, and gelatin in a ratio of 25:30:30:15 by weight, which may be dry-blended. In a related specific embodiment, immunogen may be added to the foregoing composition to form an immunogenic composition; for example, and not by way of limitation, formalin-inactivated influenza virus may be added to the adjuvant composition, e.g. at a concentration of about 0.4 percent by weight.

In additional non-limiting embodiments of the invention, microcrystalline cellulose may be suspended in solvent (aqueous or non-aqueous), vacuum-dried, then resuspended in a physiologically acceptable solvent, and the resulting solution centrifuged to remove large particles. The resulting supernatant may then be used as an immune adjuvant (see Section 8, supra). In a specific, non-limiting embodiment of the invention, 1 g microcrystalline cellulose may be suspended in 800 microliters of water, vacuum dried at

700 mmHg overnight, and then 100 mg may be suspend d in 1 ml of H_2O. This solution may then be centrifuged at 3000 rpm for 10 minutes, and the resulting supernatant decanted. Ratio of immunogen to such a supernatant adjuvant may preferably be about 500 micrograms per milliliter. An adult human dose of such a composition may preferably be about 500 microliters, but is not so limited.

4.2. METHODS OF VACCINE ADMINISTRATION

The present invention provides for a method of enhancing an immune response toward an immunogen in a subject comprising administering to the subject an effective amount of immunogen together with an effective amount of an adjuvant composition comprising microcrystalline cellulose, as described supra. An effective amount of immunogen is defined herein as that amount of immunogen which, when administered to a subject, results in the formation of antibodies directed toward the immunogen, and which, when administered with the adjuvant of the invention, results in antibody titers that confer at least partial protective immunity toward the immunogen. An effective amount of adjuvant, as used herein, is that amount of adjuvant that results in an antibody titer that is either at least about fifty percent greater than the titer obtained when immunogen is administered in the same way but without adjuvant or a duration of peak titer that is increased by at least about 20 percent over the duration obtained when immunogen is administered in the same way but without adjuvant.

According to the invention, the microcrystalline cellulose-based adjuvant composition may be administered to a subject (which may be human or non-human) via any route, including, but not limited to,

orally, intraperitoneally, intranasally, intravenously, intrathecally, or, preferably, intramuscularly or subcutaneously.

The composition may be administered as a suspension, for example, as aqueous suspension, or as a sustained release formulation. In sustained release formulations, the adjuvant composition may be comprised in microspheres or microcapsules, gelcaps, tablets, granules, beads, seeds and/or may be incorporated in an inert substrate, such as wax.

The amount of adjuvant administered may vary from subject to subject and among immunogens. In preferred, specific, non-limiting embodiments of the invention, the dosage of microcrystalline cellulose-based adjuvant may be about 1-5 milligrams per kilogram body weight.

According to preferred embodiments of the invention, immunogen may be mixed with the microcrystalline cellulose-based adjuvant composition and administered as a mixture. Alternatively, the adjuvant and immunogen may be administered separately.

Adjuvant, in conjunction with an immunogen, may be administered as a series of immunizations, if a single immunization is insufficient to produce satisfactory antibody levels.

5. EXAMPLE: CELLULOSE-BASED ADJUVANT AUGMENTED ANTIBODY TITERS TO INFLUENZA A VIRUS

5.1. MATERIALS AND METHODS

5.1.1. VACCINE FORMULATION

Dry cellulose acetate, micro-crystalline cellulose, sucrose, starch and gelatin in a ratio of 20:10:30:30:10 (w/w) were pulse blended. Two mg of the antigen, in this case formalin inactivated influenza virus A/Udorn/307/72 (H3N2), BK6, Egg3,

Clone 3A, was then added with 360 μl of water for every 500 mg of the dry mix. The wet mass was dried under vacuum to 5% water weight, then pulse blended, to form a powder that was later resuspended for immunizations. The procedure was carried out at 4°C and the preparation stored at 4°C until use.

5.1.2. IMMUNIZATION

The efficacy of the adjuvant was then tested in 6-8 week old female BALB/c mice (5/group) which were given a single, subcutaneous injection of 12.5 mg of formula containing 50 μg of inactivated influenza A virus in 100 μl of phosphate buffered saline pH 7.4. Control mice were given a single, subcutaneous injection of 50 μg of inactivated influenza A virus in saline alone.

5.1.3. MEASUREMENT OF ANTIBODY TITERS

On days 14 and 28, the mice were bled and the immune response evaluated by assaying serum immunoglobulin in an ELISA assay. ELISA assay plates were coated with virus blocked with 1% bovine serum albumin in borate saline prior to the addition of the serially diluted test specimens. After incubation, the total immunoglobulin response was measured using goat anti-mouse immunoglobulin, followed by alkaline phosphatase conjugated rabbit anti-goat antibody. Para-nitrophenyl phosphate was used as substrate and color development was measured at 405 nm after the reaction was stopped by addition of 2N NaOH. The serum hemagglutination inhibition titer was performed with mouse sera diluted 1:5 with phosphate buffered saline and treated to remove non-specific inhibitors (heated at 56° for 30 minutes, incubated with 25 percent acid-treated kaolin for 30 minutes, and

incubated with a 10 percent suspension of chicken red blood cells for 30 minutes). Two-fold dilutions of sera were prepared in 96-well microtitre plates. Viral suspension (8 HA units in an equal volume) was added to each well and incubated at room temperature for 30 minutes. A 0.5 percent suspension of chicken erythrocytes was added to each well and incubated at room temperature for 45-60 minutes. The HI titers were expressed as the reciprocal of the highest dilution that completely inhibit hemagglutination of erythrocytes. The results of both assays are presented as end-point titers.

5.2. RESULTS

Significantly higher serum immunoglobulin and hemagglutination inhibition titers were observed in mice immunized with virus prepared with cellulose acetate and microcrystalline cellulose compared with those mice that were immunized with virus in saline alone (Table I). On day 28 after immunization, the animals injected with 50 μg of whole formalin-inactivated influenza virus and cellulose-based adjuvant had an ELISA titer of 2,048,000 as compared to 128,000 for mice immunized with inactivated whole virus in saline. The hemagglutination inhibition titer for virus plus cellulose-based adjuvant was also enhanced, being 640 on day 28 compared to 40 for inactivated influenza virus in saline (Table II).

The experiment was extended through day 56 for the test groups to determine if the immune response was sustained (Tables I & II), and the maintenance of the high titers confirmed that the enhanced response was not transitory.

TABLE I
ELISA Titer

FORMULATION (50 μg of virus per 100 μl dose)	DAY AFTER IMMUNIZATION			
	14	28	42	56
CA+MC+SU+ST+G	512,000	2,048,000	2,048,000	2,048,000
SALINE	64,000	128,000	NT	NT

CA = Cellulose acetate
MC = Microcrystalline cellulose
SU = Sucrose
ST = Starch
G = Gelatin

TABLE II
Hemagglutination Inhibition Titer

FORMULATION (50 μg of virus per 100 μl dose)	DAY AFTER IMMUNIZATION			
	14	28	42	56
CA+MC+SU+ST+G	160	640	640	640
SALINE	40	40	NT	NT

CA = Cellulose acetate
MC = Microcrystalline cellulose
SU = Sucrose
ST = Starch
G = Gelatin

6. EXAMPLE: MICROCRYSTALLINE CELLULOSE EXHIBITS ADJUVANT ACTIVITY

To identify the particular component of the preparation that was responsible for immunopotentiation, a second experiment was carried

out in which groups of mice were immunized with variations on the basic preparation, each lacking one or more of the ingredients. Mice were immunized as described in Experiment 1, and the efficacy of the response determined by ELISA (Table III) and hemagglutination inhibition (Table IV) assays as described.

The formula containing only sucrose, starch and gelatin did not enhance the immune response, confirming that these are not the active ingredients. The highest serum ELISA titers were observed using the complete formula or the formula containing only microcrystalline cellulose as an active ingredient.

TABLE III
ELISA Titer

DAY AFTER IMMUNIZATION	FORMULATION (50 μg of virus per 100 μl dose)			
	(A) CA+MC+ SU+ST+G	(B) CA+SU+ ST+G	(C) MC+SU +ST+G	D SU+ST+G
0	8,000	8,000	8,000	8,000
14	32,000	64,000	64,000	64,000
28	252,000	252,000	512,000	256,000
42	1,024,000	512,000	1,024,000	256,000
56	1,024,000	512,000	1,024,000	256,000

A = Cellulose acetate: Microcrystalline cellulose: Sucrose: Starch: Gelatin (20:10:30:30:10)
B = Cellulose acetate: Sucrose: Starch: Gelatin (30:30:30:10)
C = Microcrystalline cellulose: Sucrose: Starch: Gelatin (25:30:30:15)
D = Sucrose: Starch: Gelatin (45:45:10)

TABLE IV
Hemagglutination Inhibition Titer

DAY AFTER IMMUNIZATION	FORMULATION (50 μg of virus per 100 μl dose)			
	(A) CA+MC+ SU+ST+G	(B) CA+SU+ ST+G	(C) MC+SU +ST+G	D SU+ST+G
0	< 10	< 10	< 10	< 10
14	10	10	10	10
28	40	40	40	20
42	80	80	80	40
56	160	160	80	40

A = Cellulose acetate: Microcrystalline cellulose: Sucrose: Starch: Gelatin (20:10:30:30:10)
B = Cellulose acetate: Sucrose: Starch: Gelatin (30:30:30:10)
C = Microcrystalline cellulose: Sucrose: Starch: Gelatin (25:30:30:15)
D = Sucrose: Starch: Gelatin (45:45:10)

7. EXAMPLE: COMPARISON OF CELLULOSE-BASED ADJUVANT WITH OTHER ADJUVANTS

The efficacy of the cellulose preparations was compared with established adjuvants including alum, complete Freund's adjuvant, and incomplete Freund's adjuvant. Mice were immunized as described in Experiment 2 and compared with mice immunized with inactivated influenza virus A in the appropriate adjuvant. The viral preparation in saline was mixed with an equal volume of complete or incomplete Freund's adjuvant (GIBCO, Grand Island, NY), or 1% alum (Sigma, St. Louis, MO). The ELISA results are presented in Table V and the hemagglutination inhibition titers in Table VI. The highest ELISA endpoint tier (4,048,000) was obtained by the formulation containing microcrystalline cellulose. Even complete Freund's adjuvant was not comparable (512,000) and microcrystalline cellulose adjuvant induced a better hemagglutination inhibition titer on day 28 than complete Freund's adjuvant (320 versus 160). Incomplete Freund's adjuvant and alum showed weak immunopotentiation compared to the other formulations.

TABLE V
ELISA Titer

FORMULATION	DAY AFTER IMMUNIZATION		
	0	14	28
A. MC+CA+SU+ST+G	8,000	256,000	512,000
B. CA+SU+ST+G	8,000	128,000	2,024,000
C. MC+SU+ST+G	8,000	512,000	4,048,000
D. SU+ST+G	8,000	128,000	128,000
ALUM	8,000	64,000	128,000
COMPLETE FREUND'S	8,000	512,000	512,000
INCOMPLETE FREUND'S	8,000	128,000	256,000

TABLE VI
Hemagglutination Inhibition Titer

FORMULATION	DAY AFTER IMMUNIZATION		
	0	14	28
A. MC+CA+SU+ST+G	< 10	40	80
B. CA+SU+ST+G	< 10	20	160
C. MC+SU+ST+G	< 10	160	320
D. SU+ST+G	< 10	20	40
ALUM	< 10	< 10	10
COMPLETE FREUND'S	< 10	80	160
INCOMPLETE FREUND'S	< 10	10	40

A = Cellulose acetate: Microcrystalline cellulose: Sucrose: Starch: Gelatin (20:10:30:30:10)
B = Cellulose acetate: Sucrose: Starch: Gelatin (30:30:30:10)
C = Microcrystalline cellulose: Sucrose: Starch: Gelatin (25:30:30:15)
D = Sucrose: Starch: Gelatin (45:45:10)

8. EXAMPLE: SUPERNATANT OF RESUSPENDED VACUUM-DRIED MICROCRYSTALLINE CELLULOSE HAS ADJUVANT ACTIVITY

When a mixture of influenza virus and microcrystalline cellulose was dried under vacuum, resuspended, and centrifuged, the resulting supernatant was found to exhibit greater immunogenic activity than a comparable mixture dried without vacuum.

In particular, a mixture of influenza virus (1.25 mg) and microcrystalline cellulose (250 mg) in 200 microliters of H_2O was either air-dried or vacuum-dried at 700 mmHg overnight at 4°C, and then 100 mg was resuspended in 1 milliliter of simulated intestinal fluid (U.S.P. x.x.i.i.) centrifuged at 3000 rpm for 10 minutes, and the resulting supernatant collected, and 100 microliters of supernatant was then administered subcutaneously to each of 5 mice. Sera was collected

at day 14 and day 28, and anti-influenza virus titers were evaluated by either ELISA or hemagglutination inhibition assay. Results were as follows:

TABLE VII
Titres

	ELISA	HI
AIR-DRIED		
Day 14	128,000	40
Day 28	512,000	40
VACUUM-DRIED		
Day 14	512,000	160
Day 28	1,024,000/2,048,000	160

The supernatant of resuspended vacuum-dried cellulose clearly appeared to exhibit greater adjuvant activity. The actual adjuvant may be a soluble component of cellulose and not cellulose itself.

9. EXAMPLE: IMMUNOGEN AND ADJUVANT MAY BE PREPARED SEPARATELY

Five groups of five mice each received the following preparations:

Group 1: Microcrystalline cellulose/influenza virus prepared by mixing 1.25 mg influenza virus and 250 mg microcrystalline cellulose in 200 microliters of water, vacuum drying as set forth supra, resuspending 100 mg of the product in 1 milliliter of simulated intestinal buffer, and then injecting 100 microliters of the resulting solution subcutaneously into each mouse.

Group 2: The solution prepared supra was centrifuged as set forth in Section 8,

supra, and 100 microliters of the resulting supernatant was injected subcutaneously into each mouse.

Group 3: Supernatant of vacuum-dried cellulose alone, to which influenza virus was added immediately prior to subcutaneous administration. The supernatant was prepared by resuspending 100 milligrams of vacuum dried microcrystalline cellulose in 1 milliliter of simulated intestinal buffer, and centrifuging as set forth _supra_. 100 microliters of the resulting supernatant and 50 micrograms of influenza virus was administered subcutaneously to each mouse.

Group 4: One hundred microliters of a solution, prepared by mixing 250 mg of microcrystalline cellulose with 200 microliters of water, vacuum drying as set forth _supra_, then resuspending 100 mg of the product in 1 milliliter of simulated intestinal buffer, was subcutaneously administered _without_ influenza virus (control).

Group 5: 50 micrograms of influenza virus in 100 microliters of simulated intestinal buffer was administered subcutaneously.

As depicted in Table VIII, _infra_, although the highest antibody titers were obtained using the microcrystalline cellulose/influenza pellet (Group 1), a substantial immune response was also observed when supernatant was administered, either supernatant obtained using a mixture of cellulose and virus (Group 2) or supernatant of cellulose alone mixed with virus prior to administration (Group 3). It would

therefore appear that it is not necessary to vacuum dry the cellulose and immunogen together, as a mixture.

TABLE VIII

Results

	ELISA TITERS			HI TITERS		
Group	Day 0	Day 14	Day 35	Day 0	Day 14	Day 35
1	64,000	1,024,000	2,048,000	<10	320	320
2	64,000	256,000	512,000	<10	160	160
3	64,000	256,000	512,000/ 1,024,000	<10	160	160
4	64,000	64,000	64,000	<10	<10	<10
5	64,000	128,000	256,000	<10	40	40

10. EXAMPLE: MICROCRYSTALLINE CELLULOSE ADJUVANT PREPARATIONS AND TETANUS TOXOID

Tetanus toxoid prepared by a standard commercial method was a kind gift of Commonwealth Serum Laboratories of Australia. Three groups of five BALB/C mice per group were immunized with different preparations of tetanus toxoid. Tetanus toxoid for Group 1 was diluted in phosphate buffered saline (PBS) and administered without adjuvant. Vaccine for Group 2 was prepared by combining tetanus toxoid with an extract from microcrystalline cellulose prepared by forming a wet mass of microcrystalline cellulose (5 grams cellulose and 4.5 ml H_20), and vacuum drying at 4°C. After dying, the composition was ground to a fine powder and washed three times by centrifugation with 10 ml H_2O. The supernate was saved and

concentrated to a volume of 400 μls. The supernate was then brought to a total volume of 500 μls with the tetanus toxoid solution such that each 100 μl dose contained 14 Lf tetanus toxoid. Vaccine for Group 3 was prepared by mixing 10 doses of the tetanus toxoid (14 Lf/dose) with 125 mg of a cellulose blend consisting of microcrystalline cellulose, sucrose, starch and gelatin at a ratio of 25:30:30:15. This mixture of adjuvant and vaccine was combined with water to form a wet mass and dried at 4°C under vacuum. Upon drying the mixture was ground to a fine powder and resuspended in 100 ml buffer (10 x 100 μl/dose).

Groups of 5 BALB/C mice were immunized subcutaneously with 14 Lf of tetanus toxoid per mouse (about 57 μg) either as a free solution of tetanus toxoid (Group 1); or mixed with supernatant from the cellulose preparation described above (Group 2); or compounded with a blend of microcrystalline cellulose as described above (Group 3). Mice were bled before immunization and at Day 14 and Day 28 after immunization. Anti-tetanus toxoid titers in these sera were evaluated by ELISA. Results obtained are presented in Table VIX.

TABLE VIX
TITERS
CELLULOSE ADJUVANT AND TETANUS

	ELISA TITERS		
GROUP	DO	D14	D28
1. Tetanus toxoid in solution	4,000	128,000	256,000
2. Cellulose extract and tetanus toxoid	4,000	128,000	1,024,000
3. Cellulose blend and tetanus toxoid	4,000	256,000	1,024,000

As shown in Table VIX, administration of tetanus toxoid mixed either with the cellulose blend (Group 3) or supernatant from microcrystalline cellulose preparation (Group 2) produced significantly higher antibody responses than free tetanus toxoid (Group 1).

Various references are cited herein that are hereby incorporated by reference in their entirety.

WHAT IS CLAIMED IS:

1. A method of enhancing an immune response toward an immunogen in a subject comprising administering, to the subject, an effective amount of immunogen together with an effective amount of an adjuvant composition comprising microcrystalline cellulose, so that the immune response in the subject is at least two-fold greater than if immunogen only had been administered to the subject.

2. The method of Claim 1 in which the immunogen comprises an immunogenic component of an influenza virus.

3. The method of Claim 1 in which the microcrystalline cellulose comprises at least ten percent of the adjuvant composition.

4. The method of Claim 1 in which the microcrystalline cellulose has a particle size of less than 250 microns.

5. The method of Claim 1 in which the microcrystalline cellulose has a particle size of less than ten microns.

6. The method of Claim 1 in which the adjuvant composition is administered subcutaneously.

7. The method of Claim 1 in which the adjuvant composition consists essentially of cellulose acetate, microcrystalline cellulose, sucrose, starch, and gelatin in a ratio, by weight, of approximately 20:10:30:30:10.

8. The method of Claim 7 in which the adjuvant composition is prepared separately from the immunogen and mixed with the immunogen prior to administration.

9. The method of Claim 1 in which the adjuvant composition consists essentially of microcrystalline cellulose, sucrose, starch, and gelatin in a ratio, by weight, of approximately 25:30:30:15.

10. The method of Claim 9 in which the adjuvant composition is prepared separately from the immunogen and mixed with the immunogen prior to administration.

11. A composition having immune adjuvant activity that consists essentially of cellulose acetate, microcrystalline cellulose, sucrose, starch, and gelatin, in a ratio, by weight, of approximately 20:10:30:30:10.

12. The composition of Claim 11 in which the microcrystalline cellulose has a particle size of less than 250 microns.

13. The composition of Claim 11 in which the microcrystalline cellulose has a particle size of less than ten microns.

14. An immunogenic composition of (i) cellulose acetate, microcrystalline cellulose, sucrose, starch, and gelatin, in a ratio, by weight, of approximately 20:10:30:30:10, and (ii) an effective amount of immunogen.

15. The composition of Claim 14 in which the microcrystalline cellulose has a particle size of less than 250 microns.

16. The composition of Claim 14 in which the microcrystalline cellulose has a particle size of less than ten microns.

17. The composition of Claim 14 in which the immunogen is an immunogenic component of influenza virus.

18. The composition of Claim 14 in which the immunogen is formalin-inactivated influenza virus.

19. The composition of Claim 18 in which the formalin-inactivated influenza virus is present at a concentration of about 0.4 percent.

20. A composition having immune adjuvant activity that consists essentially of microcrystalline cellulose, sucrose, starch, and gelatin in a ratio, by weight, of approximately 25:30:30:15.

21. The composition of Claim 20 in which the microcrystalline cellulose has a particle size of less than 250 microns.

22. The composition of Claim 20 in which the microcrystalline cellulose has a particle size of less than ten microns.

23. An immunogenic composition consisting essentially of (i) microcrystalline cellulose, sucrose, starch, and gelatin in a ratio, by weight, of

approximately 25:30:30:15 and (ii) an effective amount of immunogen.

24. The composition of Claim 23 in which the microcrystalline cellulose has a particle size of less than 250 microns.

25. The composition of Claim 23 in which the microcrystalline cellulose has a particle size of less than ten microns.

26. The composition of Claim 23 in which the immunogen is an immunogenic component of influenza virus.

27. The composition of Claim 23 in which the immunogen is formalin-inactivated influenza virus.

28. The composition of Claim 23 in which the formalin-inactivated influenza virus is present at a concentration of about 0.4 percent.

29. An adjuvant composition prepared by a method comprising:

a) solubilizing microcrystalline cellulose;
b) drying the microcrystalline cellulose under vacuum;
c) resuspending the vacuum-dried microcrystalline cellulose in a physiologically acceptable solvent;
d) centrifuging the resuspended microcrystalline cellulose; and
e) collecting the supernatant of the centrifuged preparation of step d),

in which the supernatant is the adjuvant.

30. A method of enhancing an immune response toward an immunogen in a subject comprising administering, to the subject, an effective amount of the adjuvant composition of claim 29, so that the immune response in the subject is at least two-fold greater than if immunogen only had been administered to the subject.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/10575

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 39/00, 9/14, 9/16, 9/18
US CL :424/88, 488, 494
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/88, 488, 494

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG
SEARCH TERMS:CELLULOSE, ADJUVANT, MICROCRYSTALLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	NATURE, VOLUME 247, ISSUED 15 FEBRUARY 1974, G.T. STEVENSON, "IMMUNISATION WITH ANTIGEN COUPLED TO AN IMMUNOSORBENT", PAGES 477-478, SEE ENTIRE DOCUMENT.	1,3-6 2
Y	US, A, 4,874,614 (BECKER) 17 OCTOBER 1989, SEE COLUMN 2, LINES 17-19 AND LINE 35.	11-16, 20-25

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 December 1993	JAN 27 1994

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